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Effect of α-Thalassemia on Sickle-Cell Anemia Linked to the Arab-Indian Haplotype in India

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Two population groups from Western India with a high prevalence of the β^S gene, one tribal (Valsad) and the other nontribal (Nagpur), were studied. The β^S gene frequency in both populations was similar (0.22 vs. 0.23), but not the clinical expression of sickle-cell anemia (SS): the sickle homozygotes in the tribal group appeared to have a mild clinical course, whereas the majority in the nontribal group exhibited a more severe clinical phenotype. Both tribal and nontribal SS patients had a similarly high mean hemoglobin (Hb)F expression (18.5% vs. 15.5%) and a high number of F cells (72.3% vs. 66.6%). DNA analysis of the β-globin gene cluster region revealed that in these two populations, this portion of DNA was identical with and corresponded to the typical Arab-Indian haplotype. Nevertheless, in heterozygotes, the mean β^S expression was lower (27.9%) in the tribal as compared to the nontribal group (35.5%). The major epistatic factor distinguishing the milder presentation in tribals vs. a more severe manifestation in nontribals was the very high frequency (0.97) of the α-thalassemia gene in the former as compared to the latter (0.24). We conclude that the phenotypic expression of sickle-cell anemia, linked to the Arab-India haplotype and expressing similar levels of HbF and F cells, is not uniformly mild in India and that α-thalassemia is a powerful and additional epistatic factor in the Indian subcontinent. Am. J. Hematol. 55:104–109, 1997. © 1997 Wiley-Liss, Inc.

Key words: sickle-cell disease; epistatic effects; genetic factors; tribals; fetal hemoglobin

INTRODUCTION

The clinical presentation of sickle-cell anemia (SS) is extremely variable between and within different population groups [1]. Although environmental factors may have a minor contributory role, there are genetic factors (higher hemoglobin (Hb)F expression, coinheritance of α-thalassemia) which help to reduce the polymerization of HbS and thereby ameliorate the severity of the disease [2–5].

In India, the β^S gene is present mainly in the tribal and in some scheduled caste (nontribal) population groups, and the frequency of heterozygotes ranges from 0–40% [6]. Earlier reports from other parts of India have shown that the disease is generally mild and associated with high levels of HbF and α-thalassemia [7,8].

A recent survey in two population groups in Western India, one tribal (Valsad) and another nontribal (Nagpur) with a high prevalence of the sickle-cell gene, revealed a diversity in clinical manifestations in the two groups. Tribals were asymptomatic or had a mild clinical presentation, while nontribals had a more severe clinical course. The present study was undertaken to answer the following questions: 1) Does the β^S mutation in the two groups have an independent origin? 2) If β^S has the same origin, are epistatic modifiers responsible for the clinical difference?

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MATERIALS AND METHODS

For 3 years we studied a total of 109 unrelated sickle-cell anaemia patients, 38 tribals from Valsad in Gujarat state, and 71 nontribals from Nagpur in Maharashtra state, ranging in age from 10–35 years. The average age in the Valsad sample was 16.8 ± 7.0 and 11.9 ± 8.0 among the Nagpur ($t = 10.4$, $P = 0.001$).

Along with this, 89 sickle heterozygous family members were studied. They included 34 AS (sickle trait) individuals from Nagpur (28 unrelated and 6 related) and 55 from Valsad (35 unrelated and 20 related).

These populations are not in geographical proximity, but both have access to similar medical care. Interviewers spoke the language corresponding to the ethnic group. Clinical data were obtained from patients' medical records.

Blood samples were collected in EDTA from all patients and their family members. Hematological indices were measured on a Baker Cell Counter 170 (Sanford, ME). Hemoglobin electrophoresis was done on cellulose acetate at pH 8.9 and quantitation of HbS was done by elution. Solubility test was used to distinguish HbS and HbD, and fetal hemoglobin was measured by HPLC [9]. HbF was quantified by classical alkaline denaturation. F cells were determined with a FACs method aided by a potent anti-γ antibody and in red cells previously fixed with formaldehyde.

A standard questionnaire was used to obtain information on all sickle cell-related symptoms, details of hospitalization, and requirements of blood transfusions. Clinical examination was done to detect splenomegaly, evidence for leg ulceration and respiratory and cardiac alterations.

DNA was extracted from 22 sickle homozygotes from Valsad and 26 sickle homozygotes from Nagpur along with available family members using a standard protocol [10]. The sickle status of all cases was confirmed by PCR followed by digestion with *Dde*I [11]. For haplotype analysis, the following restriction enzyme sites of the β-globin gene cluster were studied by PCR: *Xmn*I, *Hinc*II, *Rsa*I, *Ava*II, and *Hinf*I, using the methods described by Sutton et al. [12]. After amplification and digestion with the respective restriction enzymes, the samples were run in a polyacrylamide gel and stained with ethidium bromide for viewing in an ultraviolet (UV) transilluminator. The *Hind*III sites for $^{G}\gamma$ and $^{A}\gamma$ genes were studied by Southern blot hybridization [13] using a γIVS2 probe. The α-globin gene mapping was done by digestion with *Bam*HI followed by Southern blot hybridization using an α gene-specific probe labeled with α-^{32}P-dCTP. Direct dsDNA sequencing for the ''β silencer'' and β-locus control region (LCR) HS2 regions was done by the dideoxy nucleotide chain termination technique of Sanger et al. [14] using the sequenase kit (USB, Cleveland, OH) and α-^{35}S-dATP. The primers and the protocol for PCR were as described earlier [15,16].

RESULTS

Clinical Profile

The clinical profiles of 109 sickle-cell anemia homozygotes from two ethnic groups are depicted in Table I. Vasocclusive crisis, in the form of acute joint, abdomen, bone, and chest pain, was significantly higher ($P < 0.001$) in the nontribal individuals from Nagpur as compared to the tribal from Valsad. Infections, usually in the form of high-grade fever (over 39°C), and involving the upper respiratory or urinary tract, were again more common among nontribal patients. The rate of hospitalization for painful crisis among the nontribals was 37/71 while it was 3/38 among the tribal patients ($P < 0.001$). Splenomegaly appeared more common in tribals (56.5%) as compared to nontribals (39.5%), but the difference did not reach significance.

Hematological Profile

The hematological indices (MCV and MCH) in sickle homozygotes were significantly lower ($P < 0.001$) among the tribals (Fig. 1a,b, respectively). The reticulocyte counts among the nontribals (Valsad) ranged from 1.3–5.2% (mean ± SD = 2.0 ± 1.3), while in tribals (Nagpur) they ranged from 1–3.5% (mean ± SD = 2.9 ± 1.4). The mean HbF percentage level among tribals was 18.5 ± 6.02 % as compared to nontribals (15.5 ± 6.73%). The mean β^S expression in heterozygotes was lower in tribals (27.9 ± 4.41%) as compared to nontribals (35.5 ± 5.77%), as expected with a higher incidence of α-thalassemia.

β-Globin Gene Cluster Haplotypes

Out of 44 chromosomes studied from tribal sickle-cell patients, 42 were linked to the typical Arab-Indian haplotype (Fig. 2). The remaining two chromosomes showed two different haplotypes, that could be explained by recombination events 5′ of the ϕ pseudogene, demonstrating that the atypical haplotypes were indeed the product of recombination. In nontribals, all β^S chromosomes studied were linked to the same typical haplotype. The ''β silencer'' was sequenced and found to be identical, $[(AT)_9T_5]$, in all cases. The repeat sequence located in the β gene cluster LCR was also sequenced and was found to be identical in the typical haplotypes but not in the atypical haplotypes, since this region is 5′ to the recombination site [20,21].

F cells were determined in 11 SS patients from Valsad and were found to be 72.3 ± 27.7, and in 15 patients from Nagpur and were found to be 66.6 ± 28.5. These differences are not significant.

TABLE I. Clinical Profile of Sickle Homozygotes in Valsad and Nagpur

	Valsad (n = 38)	Nagpur (n = 71)	Chi-square value	*P* value
Rate of painful crisis				
0	18/38	12/71		
1–2	11/38	23/71	11.52	$P < 0.001$
2–10	5/38	27/71		
>10	4/38	9/71		
Hospitalization				
0	35/38	44/71		
1–3	2/38	25/71	11.26	$P < 0.001$
>3	1/38	2/71		
Leg ulcers	0/38	0/71		
Chest syndromes				
0	34/38	66/71		
1–2	2/38	5/71	0.99	Not significant
>2	2/38	0/71		
Priapism	0/38	1/71		Not significant
Aseptic necrosis (femur)	1/38	1/71		Not significant
Infections				
0	32/38	22/71		
1–3	6/38	42/71	28.05	$P < 0.0001$
>3	0/38	7/71		

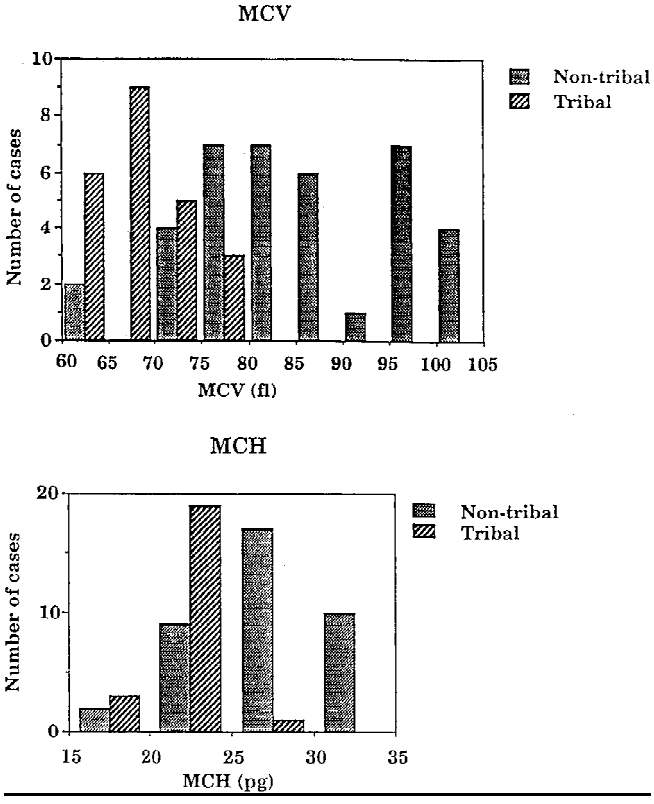

Fig. 1. MCV (a) and MCH (b) indices among SS patients in both populations.

α-Thalassemia

The prevalence of α-thalassemia in these two populations is shown in Table II. In the tribal population, the α-thalassemia haplotype is almost fixed (0.97), whereas it reaches only a value of 0.24 in the nontribal population. Of 22 SS homozygous individuals studied among tribals, 18 of them were homozygous for α-thalassemia ($-\alpha/-\alpha$) and 4 were heterozygous for α-thalassemia ($-\alpha/\alpha\alpha$), whereas in nontribals, out of 19 SS homozygous cases available for analysis, 6 were heterozygous for α-thalassemia ($-\alpha/\alpha\alpha$) and 13 had a normal α-globin gene structure ($\alpha\alpha/\alpha\alpha$). The frequency of the $-\alpha$ haplotype is also depicted in Table II.

α-Globin Gene Status and HbS Expression in AS Individuals

In heterozygotes for β^S, HbS expression was reduced in accordance with the number of α-genes (Fig. 3). Nevertheless, among AS individuals from Nagpur with the $\alpha\alpha/\alpha\alpha$ haplotype, there were individuals with low MCV, low HbA_2 (Fig. 4), and low hemoglobin, features that could be secondary to iron deficiency.

DISCUSSION

The β^S mutation is one of the commonest single-gene mutations in man and has a very widespread geographical distribution including most of Africa, the Middle East, India, and parts of the Mediterranean [17]. This mutation appears to have arisen independently in different parts of the world in different genetic backgrounds [18] and it has been found that the β^S genes are in linkage disequilibrium with at least five different restriction haplotypes in the β-globin gene cluster [18–20]. In Africa, four haplotypes are linked to β^S, designated Senegal, Benin, Bantu, and Cameroon. A fifth haplotype (the Arab-Indian) is observed in eastern Saudi Arabia and on the Indian subcontinent.

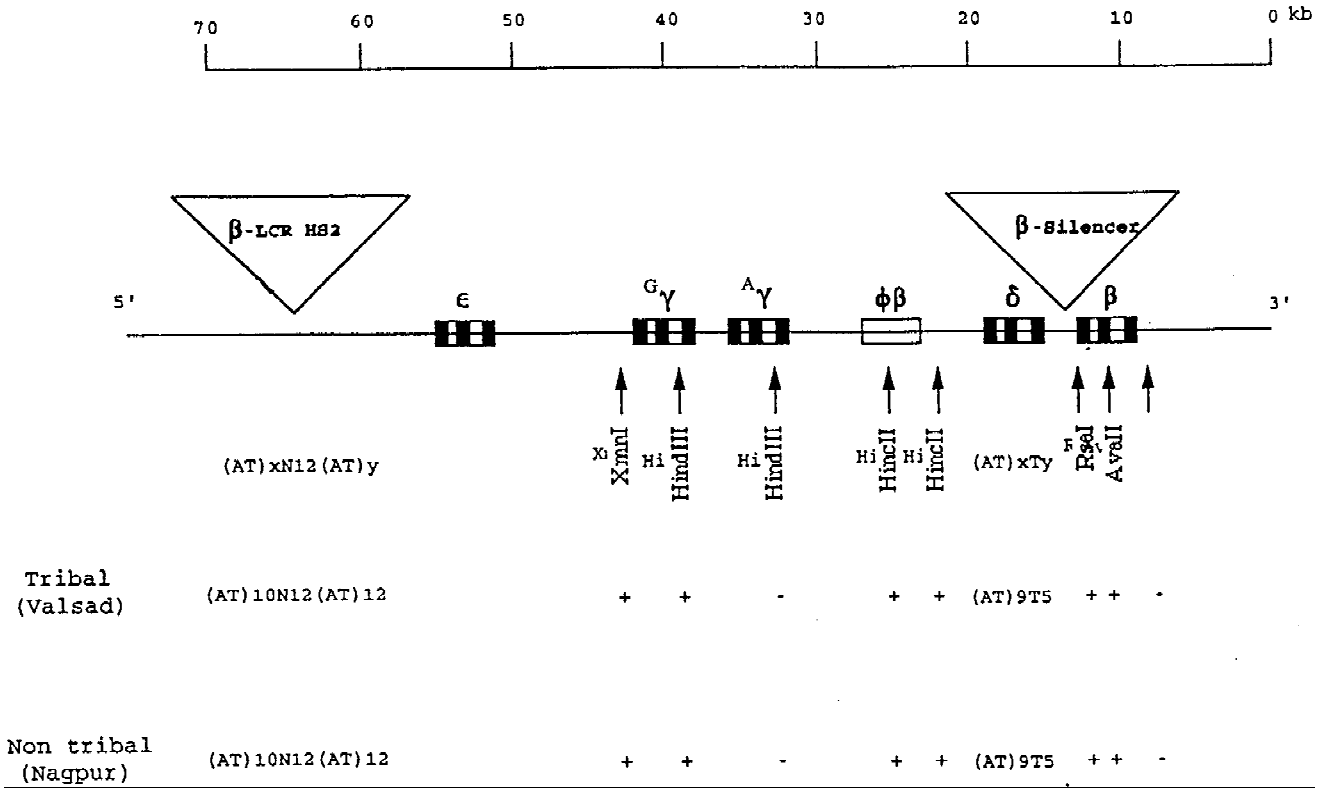

Fig. 2. β gene cluster analysis. Diagram depicts the locus control region (LCR), and the ε, $^{G}\gamma$, $^{A}\gamma$, φβ, and β genes. In addition, note the polymorphic regions in the LCR and 5′ to the β gene. Arrows indicate digestion sites for restriction enzymes. Below: Results of restriction enzyme digestion, as well as the polymorphic sequences found.

The clinical course of homozygous sickle-cell disease varies from death in early childhood to absence of serious clinical complications and a near-normal life expectancy [1]. An important determinant of clinical severity is the level of fetal hemoglobin (HbF) which inhibits intracellular polymerization of sickle hemoglobin [2]. High HbF levels are common among Indian SS patients and the disease has a very mild clinical course, according to the available reports [8].

We report here on two population groups, with different clinical presentations but equally high HbF levels, which defy the above characterization. The question is, what are the mechanisms involved in the different phenotypic presentation?

First, we studied the β gene cluster region and the β^S gene in both groups and determined that they were linked to the typical Arab-Indian haplotype. This indicates a common origin of the HbS gene in the two populations and further supports the previous observation that the sickle-cell gene in India has a unicentric origin [21]. Both groups have the same levels of HbF and F cells, and hence differences in the inhibition by HbF of the polymerization of HbS can be discounted.

Another possibility was the presence of polymorphisms within the Arab-Indian haplotype in each of these populations. Nevertheless, both groups showed the same ''β silencer motif'' $[(AT)_9T_5]$ which has been observed previously in Indians bearing the β^S gene [15]. Similarly, in both populations, the β-LCR contained the typical polymorphic sequence $[(AT)_{10}N_{12}(AT)_{12}]$ which was previously described in Indian sickle-cell anemia patients [16] and that is known to be polymorphic among different β^S gene-linked haplotypes. Thus, analysis of the β-globin gene cluster does not explain the difference in clinical expression.

We turn now to the nonlinked epistatic effects. The prevalence of α-thalassemia in the tribal SS population was 97% as compared to 24% in the nontribal population, and most of the SS patients from the tribal group have only two α genes.

Our results concur with other reports of a high prevalence of α-thalassemia among Indian tribes [8,21,23]. The effect of reduced α-globin production is manifested by lower MCV, MCH, and HbS, which is likely to diminish intravascular sickling [22]. In our study it was shown clearly that in tribals, the MCV, MCH, and HbS levels are reduced compared to nontribals. Kolozik et al. [20] also found lower β^S expression in the population of Orissa, another state in Eastern Indian where the frequency of α-thalassemia was high [23].

Finally, previous reports [15] have interpreted the low proportion of HbS in sickle trait individuals bearing the

TABLE II. Prevalence of α-Thalassemia in Nagpur and Valsad Populations*

Population	−α/−α (n)	−α/αα (n)	αα/αα (n)	αα/ααα (n)	−α/frequency (% α-thalassemics) No. of chromos.	%
Nagpur	1	10	33	1	12 (11)	13.3 (24.4)
Valsad	50	15	2	0	115 (65)	85.8 (97.0)

*chromos., chromosomes. Numbers in parentheses are frequencies of patients with α-thalassemia.

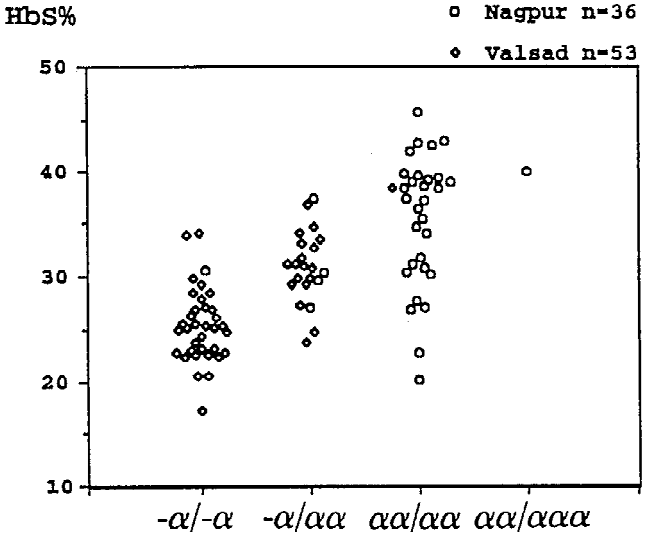

Fig. 3. Fraction of HbS according to the number of α genes in individuals heterozygous for β^S.

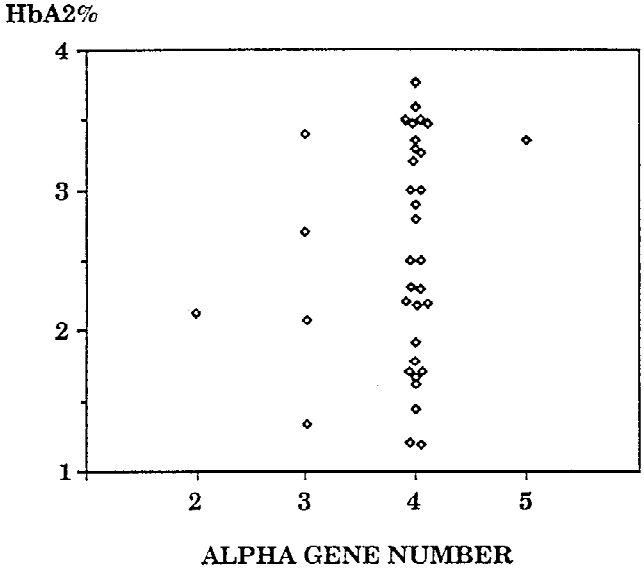

Fig. 4. Level of HbA_2 according to the number of α genes in AS individuals in Nagpur.

Arab-Indian haplotype as evidence of the presence of an active silencer for the β^S gene. Our sample of the Nagpur population could be useful in confirming or denying this interpretation, since it contains a significant number of non-α-thalassemia SS individuals with the Arab-India haplotype. Unfortunately, we observed (Fig. 3) that some AS individuals with the αα/αα haplotype had a normal proportion of HbS, while 33% had a low proportion. Nevertheless, this low proportion of HbS could be the result of an active silencer or alternatively of nondeletional α-thalassemia (unknown frequency in tribals) or, more likely, iron deficiency. The last possibility is made more likely by the presence of low MCV, low HbA_2 (Fig. 4), and low hemoglobin among the low HbS percentage AS individuals. Hence, full interpretation of these data requires therapeutic trials with iron, in a context where not all individuals heterozygous for the Arab-India haplotype have a low proportion (below 30%) of HbS, as expected with an active silencer for the β^S gene.

It is of interest that there was no difference in the incidence of acute chest syndrome, but the low incidence of this complication in both groups makes the lack of difference noninterpretable. Another point of interest in the significantly lower incidence of painful crises in the Valsad sample in association with the high incidence of α-thalassemia is in apparent contradiction with the data in the US pertaining to the β^S gene linked to African haplotypes. Billett et al. [24] were the first to report an increase in painful crisis incidence in SS patients with coexisting α-thalassemia. This has now been confirmed in a larger sample [25]. The interpretation of this phenomenon by Kaul et al. [26] is that it is the consequence of an *increased* number of SS red cells capable of adherence (deformable SS cells). If the Indian finding is confirmed in a larger sample, a possible explanation might be an overriding effect of very high HbF, particularly if some or most F cells are not adherent, i.e., resembling normal red cells. Further data are needed on this subject.

We conclude that tribal Indians from Valsad have a propensity to express high HbF levels and F cells but also a very high frequency of coinherited α-thalassemia, factors that play a *synergistic* role in ameliorating the severity of the disease. On the other hand, nontribals have a clinical phenotype comparable with homozygous sickle-cell disease but which is considerably less severe than in some African SS patients. This intermediate position may be attributed to the favorable high HbF and F cell levels and the moderate levels of α-thalassemia.

Our study of scheduled castes with moderately severe sickle-cell anemia in India should dispel the notion that sickle-cell disease in India is universally benign. Our study also shows that the clinical presentation seems to be greatly influenced by α-thalassemia in individuals bearing the Arab-Indian haplotype.

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